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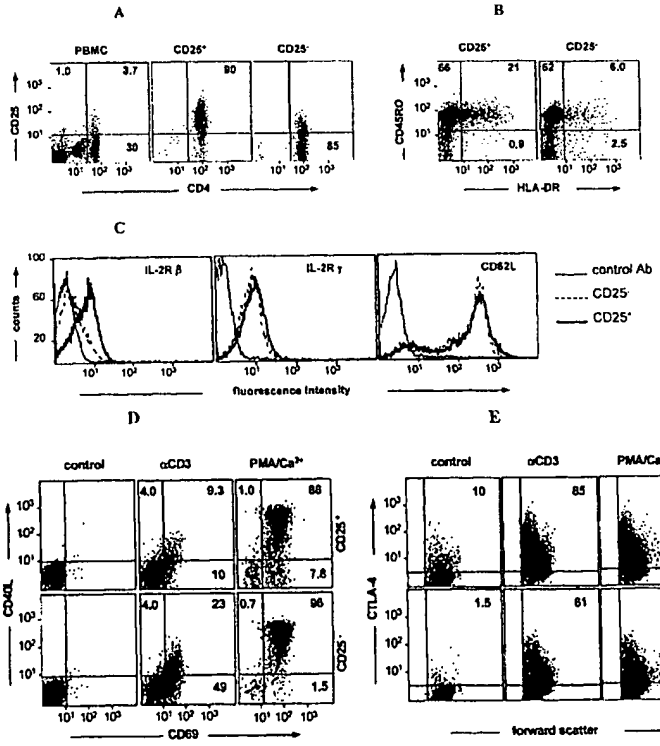

(57) Abstract: Ex-vivo isolated human CD25+CD4+ T regulatory cells, CD25+CD4+ Tr cell clones derived therefrom, a method of isolating clones and the use of ex-vivo isolated human CD25+CD4+ Tr cells or cell-clones as immunomodulators, immunosuppressive agents or for the identification of molecules that modulate the immune response.

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EX-VIVO ISOLATED $CD25^+CD4^+$ T CELLS WITH IMMUNOSUPPRESSIVE ACTIVITY AND USES THEREOF

The present invention provides ex-vivo isolated human $CD25^+CD4^+$ T regulatory (Tr) cells, homogeneous clonal populations derived therefrom with enhanced suppressive activity and their uses in the regulation of immune responses and for the identification and characterization of suppressor T cell specific molecules. More specifically, the invention is directed to the use of polyclonal $CD25^+CD4^+$ Tr cells or of homogeneous clonal $CD25^+CD4^+$ Tr cells generated ex-vivo to prevent or treat conditions where a down-regulation/suppression of the immune response is required, such as graft-vs-host disease (GvHD), organ rejection, gene therapy and autoimmune diseases, or for the identification and characterization of molecules involved in the regulation of the immune response.

BACKGROUND OF THE INVENTION

T regulatory (Tr) cells have a key role in the maintenance of immune tolerance to both self and harmless foreign antigens. Many subsets of Tr cells have been described and recently much progress has been made in understanding their ontogeny, function and mechanisms of action (reviewed in (1)). Some Tr cells do not produce cytokines and suppress T-cell responses via a mechanism that requires direct cell-cell contact (2, 3). Other subsets of Tr cells produce immunoregulatory cytokines, such as IL-10 and TGF-β, and exert their suppressive functions at least in part via the effects of these cytokines (4-8).

$CD4^+$ Tr cells that constitutively express the IL-2R α chain (CD25) have been identified in the mouse (reviewed in (2, 3)). These $CD25^+CD4^+$ Tr cells show a remarkable suppressive capacity both *in vitro* and *in vivo*. Transfer of these Tr cells reduces the pathology of experimentally-induced autoimmune

diseases such as thyroiditis, gastritis, insulin-dependent diabetes mellitus and colitis (9-12) and of experimentally induced GvHD (31), whereas depletion of $CD25^+CD4^+$ Tr cells results in the development of systemic autoimmune diseases (11, 13, 14).

Murine $CD25^+CD4^+$ Tr cells are anergic when stimulated *in vitro* with anti-CD3 mAbs, but proliferate upon addition of exogenous IL-2 (15, 16). After TCR-mediated stimulation, $CD25^+CD4^+$ Tr cells suppress the activation and proliferation of other $CD4^+$ and $CD8^+$ T cells in an antigen non-specific manner (16, 17) via a mechanism that requires cell-cell contact and that, in most systems, is independent of production of immunosuppressive cytokines (15, 16). Murine $CD25^+CD4^+$ Tr cells constitutively express cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) (9), a negative regulator of T-cell activation, and expression of this molecule is required for the ability of these cells to suppress immune responses *in vivo* (10, 18). In addition, $CD25^+CD4^+$ Tr cells may act by down-regulating the expression of CD80 and CD86 on APCs (19), although some reports suggest that APCs are not required for their suppressive activity and indicate that direct T-T cell interaction is involved (17).

DESCRIPTION OF THE INVENTION

This invention is based on the findings that human $CD25^+CD4^+$ Tr cells with immunosuppressive effects can be isolated from peripheral blood and expanded *in vitro* without loss of function, and that human $CD25^+CD4^+$ Tr cells constitute a heterogenous population from which different cell clones exhibiting suppressive or non-suppressive activity can be derived and isolated based on expression of CD25. Isolated human $CD25^+CD4^+$ Tr cells and $CD25^+CD4^+$ Tr cell clones can be used as immunosuppressive agents for the prevention or treatment of pathologies where a reduction of the immune response is desired. Typically, they will be used to prevent GvHD, organ

rejection, immune responses directed against foreign proteins introduced during gene therapy and autoimmune diseases, especially type 1 diabetes. $CD25^+CD4^+$ Tr cells isolated from peripheral blood can be stimulated and cultured in vitro, allowing for the possibility to select and expand antigen-specific suppressor T cells. Expanded $CD25^+CD4^+$ Tr cells maintain their regulatory capacity in vitro, and thus can be used to regulate T cell responses in vitro, whereas both freshly-isolated and in vitro-expanded human $CD25^+CD4^+$ Tr cells can be utilized in therapy in vivo. The methods and conditions for isolation and in vitro expansion of $CD25^+CD4^+$ Tr cells are described in detail in the section Materials and Methods. Essentially, freshly-isolated human $CD25^+CD4^+$ Tr cells can be expanded in vitro under one or more of the following conditions: co-culture with feeder-cell mixture, polyclonal stimulation, antigen specific stimulation, addition of cytokines.

The T cells thus obtained can be re-introduced in the patient. The preferred modalities under which $CD25^+CD4^+$ Tr cells are used in therapy or prophylaxis will depend on the particular condition to prevent/treat.

For example, to prevent/treat GvHD, $CD25^+CD4^+$ Tr can be isolated from leukapheresis of the bone-marrow donor, frozen if necessary, and administered to the recipient at the time of transplant, prior to the transplant, or in the subsequent months. Alternatively, $CD25^+CD4^+$ Tr cells from the donor could be stimulated with host APC in vitro, in order to generate and expand alloantigen-specific $CD25^+CD4^+$ Tr cells that would specifically suppress host-specific responses in vivo.

To prevent/treat organ rejection, $CD25^+CD4^+$ Tr cells can be isolated from the recipient, frozen if necessary, and administered prior to transplant, at the time of transplant or in the subsequent months. Alternatively, $CD25^+CD4^+$ Tr cells could be stimulated in vitro with autologous APCs that have been co-cultured with tissue from the organ in question and will

therefore present foreign antigens through the indirect pathway (32). The resulting $CD25^+CD4^+$ Tr cell lines would be specific for antigens expressed by the transplanted organ and could be used to suppress organ-specific responses in vivo.

To prevent autoimmune diseases, bulk populations of autologous $CD25^+CD4^+$ Tr cells can be isolated and re-infused. Alternatively, antigen-specific $CD25^+CD4^+$ Tr cells could be expanded in vitro by stimulation with autologous APCs and self-antigens derived from tissues which are targets of the disease. Upon re-administration of the in vitro-expanded $CD25^+CD4^+$ Tr cells, they will suppress anti-self responses in vivo.

To prevent immune response in gene therapy, $CD25^+CD4^+$ Tr cells can be isolated from the recipient, and cells which are specific for antigens encoded by the therapeutic vector could be expanded in vitro by stimulation with transduced autologous APCs expressing the transgene. These cells can be frozen if necessary, and administered at the time of gene therapy treatment or in the subsequent months.

Advantageously, a homogeneous clonal population of $CD25^+CD4^+$ Tr suppressive cells is used for the therapeutic applications indicated above. The method for isolating suppressive $CD25^+CD4^+$ Tr clones essentially comprises the steps of:

a) purifying $CD4^+$ T cells from PBMCs;

b) separating $CD25^+$ from $CD25^-$ T cells;

c) cloning $CD25^+CD4^+$ T cells by limiting dilution;

d) stimulating with phytohemagglutinin (PHA) or anti-CD3 mAb in the presence of IL-2;

e) selecting the cell clones that display a constitutively high expression of CD25.

According to step (a), $CD4^+$ cells can be purified by positive selection

with anti-CD4-coupled microbeads. Step (b) can be carried out by marking $CD25^+$ cells using labelled anti-CD4/25 monoclonal antibodies and purifying $CD25^+$ cells by FACS-sorting. The clones obtained from step (c), which can be maintained in X-vivo 15 culture-medium or in other cellular media, supplemented with 5% pooled or autologous human serum, are preferably re-stimulated by co-culture with feeder-cell mixture, by antigens or by cytokines, more preferably by an allogenic or autologous feeder-cell mixture consisting of irradiated PBMCs, with or without irradiated autologous or allogeneic EBV-transformed cell lines (eg. JY). Suppressive clones which display a constitutively high expression of CD25 can be selected, according to step (d), on the basis of the following characteristics:

- 100% constant-positivity for CD25 expression in the resting phase at least 10 days after stimulation with phytohemagglutinin or anti-CD3 mAb in the presence of IL-2;
- expression of CD25 at a significantly higher level in comparison to T cell clones isolated in parallel from $CD25^-CD4^+$ T cells or non suppressive clones isolated from $CD25^+CD4^+$ T cells.

At the end of steps (a)-(d), homogeneous $CD25^+CD4^+$ T-cell clones constitutively expressing CD25, anergic and with high suppressive capacity are isolated. The suppressive clones, in contrast to the non-suppressive ones, are characterized by significant production of TGF-β and no production of IL-2.

In a further embodiment, the invention provides an immunosuppressive agent containing isolated $CD25^+CD4^+$ Tr-cells and/or $CD25^+CD4^+$ Tr cell clones constitutively expressing CD25, and optionally other active substances, such as cytokines, or other immunosuppressive proteins. Preferably the immunosuppressive agent will be in the form of a stabilized cell preparation.

Besides the envisaged clinical applications, the $CD25^+CD4^+$ Tr

suppressive cell clones can be used to set up systems in vitro for the identification of molecules that modulate the immune response, in particular suppressor-T-cell-specific molecules. According to a preferred embodiment, such $CD25^+CD4^+$ Tr cell clones will be used in large scale gene expression arrays, in differential proteomics screenings and in the generation of monoclonal antibodies specific for Tr cells constitutively expressing CD25. These applications are greatly aided by the homogeneity of comparative samples, such as that provided by cell populations of clonal origin.

DESCRIPTION OF THE FIGURES

Figure 1. *Isolation and cell-surface phenotype of human $CD25^+CD4^+$ Tr cells.* $CD4^+$ T cells were isolated from PBMCs, and separated into $CD25^+$ and $CD25^-$ fractions. Purity (**A**) and expression of CD45RO, HLA-DR (**B**), IL-2Rβ, IL-2Rγ and CD62L (**C**) was determined by FACS. $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were either cultured in medium alone, or activated with immobilized anti-CD3 mAbs or PMA and calcium ionophore for 6 hours (**D**) or 24 hours (**E**). Cells were analyzed for surface-expression of CD40L and CD69 (**D**), and for intracytoplasmic expression of CTLA-4 (**E**). Results are representative of 6 independent experiments.

Figure 2. *$CD25^+CD4^+$ Tr cells are anergic and suppress proliferation to alloantigens.* Purified $CD25^+CD4^+$ Tr cells (100,000 cells/well) were tested for their ability to proliferate in response to immobilized anti-CD3 mAbs (**αCD3**) (10μg/ml) in the absence or presence of soluble anti-CD28 mAbs (**αCD28**) (1μg/ml), secondary rabbit anti-mouse Abs (**αCD28 CL**) (10μg/ml), and/or IL-2 (100U/ml). After 72 hours of culture, ^{3}H-thymidine was added for an additional 16 hours (**A**). $CD25^-CD4^+$ T cells (50,000 cells/well) were tested for their ability to proliferate in response to allogeneic APCs in the absence or presence of increasing numbers of autologous $CD25^+CD4^+$ Tr cells (**B**). $CD25^-CD4^+$ T cells were activated to induce CD25 expression. After 48

hours T cells that became $CD25^+$ ($\mathbf{CD25^{+i}}$) were purified and tested for their ability to suppress proliferation of $CD25^-$ T cells in response to alloantigens (**C**). $CD25^-CD4^+$ T cells were activated by alloantigens with or without $CD25^+CD4^+$ Tr cells (1:1 ratio) in the presence of the indicated mAbs (10μg/ml). Numbers represent the percent reduction in proliferation compared to culture in the absence of $CD25^+CD4^+$ Tr cells (**D**). For **B-D**, after 96 hours, ^{3}H-thymidine was added for an additional 16 hours. Results are representative of 6 independent experiments for **A**, 9 for **B** and 3 for **C&D**.

Figure 3. *Expansion and cell-surface phenotype of $CD25^+CD4^+$ Tr cells.* $CD25^+$ and $CD25^-CD4^+$ T cells were purified, and activated with anti-CD3 mAbs, allogeneic feeder-cell mixture and exogenous IL-2. Cells were split as necessary and after 2 weeks were analyzed by FACS for expression of CD25 and CD4 (**A**). In parallel, cells were analyzed for cell-surface expression of CD40L and CD69 following activation for 6 hours with immobilized anti-CD3 mAbs or PMA and calcium ionophore (**B**). Constitutive levels of CTLA-4 expression was determined by intracytoplasmic staining (**C**). Results are representative of 3 independent experiments.

Figure 4. *Cultured $CD25^+CD4^+$ Tr cells retain their suppressive capacity.* $CD25^+$ and $CD25^-CD4^+$ T cells were purified and activated with anti-CD3 mAbs, allogeneic feeder-cell mixture and exogenous IL-2. After 14 days of culture, T cells were tested for their ability to proliferate in response to anti-CD3 mAbs (10μg/ml) in the absence or presence of soluble anti-CD28 mAbs (1μg/ml) and/or IL-2 (100U/ml) (**A**). Cultured $CD25^-CD4^+$ T cells (50,000 cells/well) were tested for their ability to proliferate in response to alloantigens in the absence or presence of increasing numbers of *in vitro*-cultured, autologous $CD25^+CD4^+$ Tr cells (**B**). Cultured $CD25^-CD4^+$ T cells were activated by allogeneic APCs (from a donor different from that used for expansion) with or without $CD25^+CD4^+$ Tr cells (1:1 ratio) in the presence of

the indicated mAbs (10μg/ml). Numbers represent the percent reduction in proliferation compared to culture in the absence of $CD25^+CD4^+$ Tr cells (**C**). For all cultures, after 48 hours, ^{3}H-thymidine was added for an additional 16 hours. Results are representative of 3 independent experiments.

Figure 5. *Isolation of human $CD25^+CD4^+$ T cells at the clonal level.* $CD4^+$ T cells were isolated from peripheral blood, stained with anti-CD4 and anti-CD25 mAbs, and separated into $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells by FACS sorting to a purity greater than 98 and 99% respectively.

Figure 6. *$CD25^+CD4^+$ T cell clones are heterogenous in terms of their expression of CD25 in the resting phase*. Resting T-cell clones were stained with anti-CD4 and -CD25 mAbs 12-14 days after the last re-stimulation, The number (#) of the T cell clone is indicated on the upper left, and the MFI and percent of CD25 positive cells is on the upper right.

Figure 7. *$CD25^+CD4^+$ T cell clones are heterogenous in term of their proliferative response to activation via the TCR*. Resting T-cell clones were tested for their ability to proliferate in response to anti-CD3 mAbs (10μg/ml) in the absence or presence of IL-2 (100U/ml). After 48 hours of culture, ^{3}H-thymidine was added for an additional 16 hours.

Figure 8. *Suppression of naive T cell responses by $CD25^+CD4^+$ T cell clones*. Autologous $CD4^+$ T cells were purified and tested for their ability to proliferate in response to anti-CD3 mAbs and irradiated CD3-depleted APCs (**A**) or anti-CD3 mAbs immobilized on plastic (**B**). After 72 (**A**) or 48 hours (**B**) of culture, ^{3}H-thymidine was added for an additional 16 hours. Numbers indicate percent reduction in proliferation in comparison to the naive $CD4^+$ T cells alone.

DETAILED DESCRIPTION OF THE INVENTION

Isolation and cell-surface phenotype of human $CD25^+CD4^+$ Tr cells

$CD25^+CD4^+$ Tr cells are present in human PBMCs. On average they

represent 3.0% (range 1.6-4.4%, n=6) of total PBMCs and 12.8% (range 9.8-18.1%, n=6) of $CD4^+$ T cells. These cells could be readily isolated, with purities greater than 90% (Figure 1A). The majority (82 ±5.1%) of freshly isolated $CD25^+CD4^+$ Tr cells also expressed CD45RO and the percentage of $CD25^+CD4^+$ Tr cells expressing HLA-DR was significantly higher than that in the $CD25^-CD4^+$ population (17.3 ±4.9% vs 6.4 ±2.9%, n=6) (Figure 1B). In addition, human $CD25^+CD4^+$ Tr cells expressed higher levels of the IL-2Rβ in comparison to $CD25^-CD4^+$ T cells (61.9 ±2.6% vs 33.1 ±2.5%, n=5) (Figure 1C) and a subset of freshly isolated $CD25^+CD4^+$ Tr cells expressed CTLA-4 (8.4 ±1.6%, n=6) (Figure 1E). In contrast, expression of the IL-2Rγ and CD62L was equivalent on both populations of T cells. $CD25^+CD4^+$ Tr cells and $CD25^-CD4^+$ T cells were $CD3^+TCR\alpha\beta^+$. Thus, human $CD25^+CD4^+$ Tr cells express markers which are characteristic of memory T cells and low constitutive levels of CTLA-4, similarly to murine $CD25^+CD4^+$ Tr cells (9, 10, 15, 17, 18).

Following TCR-mediated stimulation, human $CD25^+CD4^+$ Tr cells expressed lower levels of activation markers in comparison to $CD25^-CD4^+$ T cells. The proportion of CD40L+ cells was 17.3 ±2.3% in $CD25^+CD4^+$ Tr cells vs 28.4 ±1.8% in the $CD25^-CD4^+$ T-cell subset (p 0.005); whereas 30.9 ±7.0% of $CD25^+CD4^+$ Tr cells vs 54 ±9.1% of $CD25^-CD4^+$ T cells expressed CD69 (p 0.006) (Figure 1D). Time course experiments demonstrated that the reduced levels of CD40L and CD69 on $CD25^+CD4^+$ Tr cells were not due to altered kinetics of expression. After activation with PMA and calcium ionophore there was no statistically significant difference between expression of CD69 or CD40L on $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells, although in general fewer $CD25^+CD4^+$ Tr cells expressed CD40L.

Following activation with anti-CD3 mAbs or PMA and calcium ionophore, the percentage of $CD25^+CD4^+$ Tr cells expressing CTLA-4 was

higher than that of $CD25^-CD4^+$ T cells. In addition, CTLA-4 expression levels were approximately 3 fold higher on $CD25^+CD4^+$ Tr cells (Figure 1E). Collectively, these data demonstrate that upon TCR activation human $CD25^+CD4^+$ Tr cells have a surface molecule expression profile which is unique and distinct from that of other $CD4^+$ T-cell subsets.

Proliferation and cytokine production by human $CD25^+CD4^+$ Tr cells

Freshly isolated $CD25^+CD4^+$ Tr cells did not proliferate in response to immobilized anti-CD3 mAbs, and addition of soluble anti-CD28 mAbs resulted in a modest and variable increase in proliferation. In contrast, crosslinked anti-CD28 mAbs completely reversed the unresponsiveness of $CD25^+CD4^+$ Tr cells to TCR activation. Addition of exogenous IL-2 partially restored the proliferation of $CD25^+CD4^+$ Tr cells in response to anti-CD3 mAbs, and proliferation was further enhanced by soluble anti-CD28 mAbs (Figure 2A). These results indicate that human $CD25^+CD4^+$ Tr cells have a specific defect in their ability to proliferate after TCR-mediated activation (15, 16).

Human $CD25^+CD4^+$ Tr cells were analyzed for their ability to produce cytokines. Following stimulation with immobilized anti-CD3 mAbs, with or without soluble anti-CD28 mAbs, no detectable levels of IL-2, IL-10, IL-4, TGF-β or IFN-γ could be measured. In contrast, when stimulated with anti-CD3 and soluble anti-CD28 mAbs in the presence of exogenous IL-2, $CD25^+CD4^+$ Tr cells produced significant levels of IL-4, IL-10, IFN-γ and TGF-β (Table 1). Under these stimulation conditions $CD25^+CD4^+$ Tr cells had a cytokine profile that was comparable to that of $CD25^-CD4^+$ T cells. In contrast, differences in cytokine production were observed following activation with allogeneic APCs. Both $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells produced IL-10, TGF-β and IFN-γ, but no IL-4. However, the striking difference between the $CD25^+CD4^+$ and $CD25^-CD4^+$ T cell populations is

that the $CD25^+$ cells failed to secrete IL-2, indicating that these cells have a specific defect in production of IL-2. Interestingly, $CD25^+CD4^+$ Tr cells consistently produced less IFN-γ upon alloantigen stimulation than did $CD25^-CD4^+$ T cells.

Human $CD25^+CD4^+$ Tr cells suppress the proliferative responses of naive $CD25^-CD4^+$ T cells to alloantigens.

We investigated the regulatory properties of $CD25^+CD4^+$ Tr cells by testing their ability to suppress the proliferative responses of naive $CD25^-CD4^+$ T cells to alloantigens. $CD25^-CD4^+$ T cells were stimulated with allogeneic APCs and increasing numbers of autologous $CD25^+CD4^+$ Tr cells were added. Addition of as few as 2,500 $CD25^+CD4^+$ Tr cells to 50,000 $CD25^-CD4^+$ T cells resulted in a reduced proliferation of $CD25^-CD4^+$ T cells. At a ratio of 1:1, $CD25^+CD4^+$ Tr cells inhibited the proliferation of naive $CD25^-CD4^+$ T cells by an average of 75.0 ±2.9% (n=9) (Figure 2B). The $CD25^+CD4^+$ Tr cells themselves failed to proliferate in response to alloantigens. Furthermore, $CD25^+CD4^+$ Tr cells suppressed the proliferation of $CD25^-CD4^+$ T cells in response to PHA in the presence of APCs (by an average of 81.2 ±5.0%, n=6) and to immobilized anti-CD3 alone (by an average of 79.4 ±14.6%, n=3). These latter data indicate that $CD25^+CD4^+$ Tr cells have a direct suppressive effect on T cells that is independent of APCs, similar to murine $CD25^+CD4^+$ Tr cells (17). In addition, $CD25^+CD4^+$ Tr cells suppressed production of IFN-γ and IL-2 by $CD25^-CD4^+$ T cells activated with anti-CD3 mAbs or allogeneic APCs.

In order to demonstrate that this suppressive capacity was an intrinsic property of T cells constitutively expressing CD25 *in vivo*, we tested whether $CD25^-CD4^+$ T cells which expressed CD25 following activation *in vitro* showed regulatory effects. To this aim, $CD25^-CD4^+$ T cells were activated with anti-CD3 and anti-CD28 mAbs, and after 48 hours the $CD25^+$ T cells

were isolated and tested for their ability to suppress freshly isolated autologous $CD25^-$ T cells. As shown in Figure 2C, T cells which became $CD25^+$ after *in vitro* activation proliferated in response to alloantigens, and enhanced rather than suppressed the response of $CD25^-CD4^+$ T cells. These data indicate that inhibition of T cell proliferation is a unique property of cells which constitutively express CD25 *in vivo*.

Several studies show that some subsets of Tr cells, such as type 1 Tr (Tr1) and Th3 cells, produce IL-10 and/or TGF-β and suppress immune responses via production of these cytokines (4-8). Since $CD25^+CD4^+$ Tr cells produced both IL-10 and TGF-β upon stimulation with allogeneic APCs (Table 1) the role of these cytokines on inhibition of allogeneic responses by human $CD25^+CD4^+$ Tr cells was investigated. As shown in Figure 2D, addition of neutralizing anti-IL-10R or anti-TGF-β mAbs had no significant effect on the ability of $CD25^+CD4^+$ Tr cells to suppress the proliferation of $CD25^-CD4^+$ T cells in response to alloantigens. In 3 independent experiments an average of 67.2 ±6.0% suppression was observed in the presence of 10μg/ml of control IgG, 63.8 ±5.8% with 10μg/ml of anti-IL-10R and 69.0 ±3.4% with 10μg/ml of anti-TGF-β mAbs. Similar results were obtained with a 5 fold higher concentration of anti-TGF-β mAbs. Addition of both anti-IL-10R and anti-TGF-β mAbs resulted in a slight, but not statistically significant, reversal of suppression mediated by $CD25^+CD4^+$ Tr cells (from 67.2 ±6.0% to 52.4 ±5.7%, p 0.06).

$F(ab')_2$ fragments from antibodies which specifically block the ability of CTLA-4 to bind to CD80/86, without affecting signals via CD28, have previously been shown to inhibit the production of TGF-β by Tr1 cells (20). Addition of the same blocking anti-CTLA-4 mAbs had no significant effect on the suppressive activity of $CD25^+CD4^+$ Tr cells. These data suggest that despite the fact that $CD25^+CD4^+$ Tr cells express high levels of CTLA-4

(Figure 1E), this molecule is not required for their suppressive activity.

Human CD25$^+$CD4$^+$ Tr cells can be expanded in vitro

We have previously shown that human Tr1 cells which have been cloned and expanded *in vitro* maintain their regulatory activity (6). Using a protocol similar to that described for Tr1 cells, we determined whether CD25$^+$CD4$^+$ Tr cells could be expanded. CD25$^+$CD4$^+$ Tr cells did not proliferate in response to anti-CD3 alone (Figure 2A), but when activated with anti-CD3 mAbs in the presence of an allogeneic feeder-cell mixture and exogenous IL-2, an expansion which was similar to that of CD25$^-$CD4$^+$ T cells was obtained (20-30 fold increase at day 14). *In vitro*-expanded human CD25$^+$CD4$^+$ Tr cells remained positive for CD25 even after culture for more than one month (Figure 3A). In contrast, all CD25$^-$CD4$^+$ T cells expressed CD25 after activation, but the expression gradually decreased with time. Persistent expression of CD25 has also been observed in murine CD25$^+$CD4$^+$ Tr cells activated *in vivo* (21).

Similar to freshly isolated CD25$^+$CD4$^+$ Tr cells, the proportion of *in vitro*-expanded CD25$^+$CD4$^+$ Tr cells expressing CD40L following activation with anti-CD3 mAbs was consistently lower in comparison to CD25$^-$CD4$^+$ T cells (12.7 ±3.3% vs 36.9 ±8.3%, p 0.01). However, in contrast to freshly isolated cells, cultured CD25$^+$CD4$^+$ Tr cells expressed normal levels of CD69 following polyclonal TCR-mediated activation (Figure 3B). Thus, reduced upregulation of CD40L is also a characteristic of expanded CD25$^+$CD4$^+$ Tr cells. As expected, cultured CD25$^+$CD4$^+$ and CD25$^-$CD4$^+$ T cells expressed similar levels of both CD40L and CD69 following activation with PMA and calcium ionophore. *In vitro*-expansion of CD25$^+$CD4$^+$ Tr cells did not alter their constitutive expression of CTLA-4, and they continued to express this inhibitory molecule at significantly higher levels than their CD25$^-$CD4$^+$ counterparts (Figure 3C).

In vitro-expanded $CD25^+CD4^+$ Tr cells remain anergic and retain their suppressive capacity

In vitro-expanded $CD25^+CD4^+$ Tr cells failed to proliferate in response to anti-CD3 mAbs alone, and proliferation could only be completely restored by addition of exogenous IL-2 (Figure 4A). Moreover, cultured $CD25^+CD4^+$ Tr cells failed to proliferate in response to alloantigens, and retained their ability to suppress the proliferation of autologous $CD25^-CD4^+$ T cells (at a 1:1 ratio, an average 64 ±3.8% (n=3) suppression was observed) (Figure 4B). These data indicate that $CD25^+CD4^+$ Tr cells expanded *in vitro* maintain their regulatory functions and behave similarly to freshly isolated $CD25^+CD4^+$ Tr cells. Since in this experiment the $CD25^-CD4^+$ responder T cells had been previously activated and expanded *in vitro*, these data demonstrate that $CD25^+CD4^+$ Tr cells suppress not only the response of freshly isolated naive $CD25^-CD4^+$ T cells, but also that of previously activated memory $CD25^-CD4^+$ T cells (Figure 4B). Finally, similar to observations with freshly isolated $CD25^+CD4^+$ Tr cells, the suppression mediated by in vitro-expanded $CD25^+CD4^+$ Tr cells was not reversed by addition of neutralizing anti-IL-10R, anti-TGF-β or anti-CTLA-4 mAbs (Figure 4C).

In the present study we show that human $CD4^+$ T cells which express CD25 in vivo are a unique subset of Tr cells. Human $CD25^+CD4^+$ Tr cells are anergic, fail to produce IL-2, constitutively express CTLA-4, and suppress the proliferation of naive $CD4^+$ T cells, as described for murine $CD25^+CD4^+$ Tr cells (2, 3). In addition, following polyclonal TCR-mediated activation, human $CD25^+CD4^+$ Tr cells strongly upregulate CTLA-4, display reduced expression of CD40L, and produce cytokines. CD25 and CTLA-4 remain constitutively expressed on in vitro-expanded human $CD25^+CD4^+$ Tr cells, while following activation, up-regulation of CD40L is still defective. More interestingly, in vitro expanded $CD25^+CD4^+$ Tr cells retain their potent

suppressive activity, even towards previously activated memory T cells. The observation that functional Tr cells can be expanded in vitro and can regulate responses of memory T cells is of great clinical relevance for the use of $CD25^+CD4^+$ Tr cells as a cellular therapy in T-cell mediated diseases.

The role of immunoregulatory cytokines in the suppression mediated by $CD25^+CD4^+$ Tr cells remains an open question. Alloantigen-activated $CD25^+CD4^+$ Tr cells did not proliferate but produced IL-10, IFN-γ and TGF-β, and indeed possessed a profile of cytokine production which is comparable to that of Tr1 cells (i.e. IL-10+IFN-γ+TGF-β+IL-4-IL-2-/low) (6). However, we observed only a slight reversal of suppression in the presence of both neutralizing anti-IL-10R and anti-TGF-β mAbs, which is consistent with previous observations that production of IL-10 and TGF-β is dispensable for the regulatory function of $CD25^+CD4^+$ Tr cells (15, 16). On the other hand, in a murine model of experimentally-induced colitis, both IL-10 and TGF-β were found to be required for suppression mediated by $CD25^+CD4^+$ Tr cells (5, 10). The basis for this discrepancy in the involvement of IL-10 and TGF-β is unclear. $CD25^+CD4^+$ Tr cells have the capacity to produce IL-10 and TGF-β, but production of these cytokines may depend on their maturation state and the environmental context in which they are activated.

Previous reports demonstrated that direct cell-cell contact is required for murine $CD25^+CD4^+$ Tr cells to exert their suppressive effects (15, 16). Despite constitutive and persistent expression of CTLA-4, anti-CTLA-4 mAbs failed to abrogate the suppressive activity of human $CD25^+CD4^+$ Tr cells. These data are in agreement with a study indicating that signals via CTLA-4 were dispensable for suppression by mouse $CD25^+CD4^+$ Tr cells in vitro (15). However, more recent reports indicate that expression of CTLA-4 is essential for suppression mediated by these cells (10, 18). It is possible that suppression of proliferation operates via mechanisms which differ depending on the

stimuli and microenvironment, or that human and mouse $CD25^+CD4^+$ Tr cells act through different mechanisms. Finally, suppression is not simply due to consumption of IL-2 as murine $CD25^+CD4^+$ Tr cells suppressed IL-2 production at the transcriptional level (15). In addition, human $CD4^+$ T cells which expressed CD25 following activation in-vitro did not suppress responses of $CD25^-CD4^+$ T cells, indicating that high expression of CD25 does not simply result in sequestration of IL-2.

Human $CD25^+CD4^+$ Tr cells expand in vitro and maintain their unique cell-surface marker profile and suppressive functions. To our knowledge, these data represent the first report of in vitro expansion of human T suppressor cell lines.

The clinical use of $CD25^+CD4^+$ T regulatory (Tr) cells can be envisaged to down-regulate undesired immune responses in a number of pathological conditions. We have shown that $CD25^+CD4^+$ Tr cells with suppressive function can be readily isolated from peripheral blood. Importantly, these cells can be stimulated and cultured in vitro, allowing for the possibility to select and expand antigen-specific suppressor T cells. Expanded $CD25^+CD4^+$ Tr cells maintain their regulatory capacity in vitro, and thus could be used to regulate T cell responses in vitro.

Isolation and characterization at the clonal level of human $CD25^+CD4^+$ T cells with suppressive capacity

In order to determine the relationship between IL-10 producing Tr1 cells (22) and $CD25^+CD4^+$ Tr cells (23, 24), we attempted to isolate $CD25^+CD4^+$ Tr cells at the clonal level. It has previously been described that only approximately 0.8% of peripheral blood mononuclear cells which are $CD4^+$ and high for $CD25^+$ have a suppressive capacity in vitro (25). We therefore purified $CD4^+$ T cells from peripheral blood, and by FACS sorting, purified $CD25^{bright}CD4^+$ Tr cells which represent approximately 2.9% of $CD4^+$ T cells

(0.6% of total peripheral blood in this donor). The resulting $CD25^-$ and $CD25^{bright}$ populations were 99 and 98% pure respectively (Figure 5). The purified cells were subsequently cloned by limiting dilution at 1 cell/well and stimulated with PHA and an allogeneic feeder cell mixture as described in the materials and methods and as previously described (26) After 8 days, one 96-well plate from each cloning was pulsed overnight with thymidine in order to determine the number of proliferating wells (the cloning efficiency). The $CD25^-CD4^+$ T cells had a cloning efficiency of 42%, whereas the $CD25^+CD4^+$ T cells had a significantly lower efficiency of only 10.2%. After 14 days, 120 $CD25^+CD4^+$ T-cell clones were picked, restimulated and expanded for analysis.

As one of the defining characteristics of $CD25^+CD4^+$ Tr cells is constitutive and high expression of CD25, we screened the $CD25^+CD4^+$ T-cell clones for expression of CD25 at least 10 days after restimulation (in the resting phase). A shown in Figure 6, the clones displayed a heterogeneous expression of CD25. Approximately half the clones remained 99-100% positive for CD25, and possessed a relatively high mean fluorescence intensity (MFI). Other clones contained a significant number of $CD25^-$ cells and a lower MFI. T-cell clones derived the $CD25^-CD4^+$ T cells displayed a low percentage of $CD25^+$ cells in the resting phase and consequently also a low MFI.

The clones were subsequently tested for their ability to proliferate in response to activation via the TCR in the absence or presence of exogenous IL-2. It has been well established that both murine and human $CD25^+CD4^+$ T cells fail to proliferate in response to αCD3 mAbs in the absence of co-stimulation via CD28 and/or addition of IL-2 (23, 24, 26). In order to determine if this was also true at the clonal level, we tested the $CD25^+CD4^+$ T-cell clones for their ability to proliferate in the presence or absence of IL-2.

Similar to the heterogeneity observed in terms of expression of CD25, the clones were also heterogeneous in terms of proliferation. The majority of the clones (58/72, 80%) were anergic and failed to proliferate in response to αCD3 mAbs, but proliferated well in the presence of IL-2. The remaining clones (14/72, 20%) proliferated well in response to αCD3 mAbs even in the absence of IL-2. A representative subset of the 72 cloned tested is shown in Figure 7. In contrast, amongst the $CD25^{-}CD4^{+}$ T-cell clones tested, the majority (14/22, 65%) proliferated well in response to αCD3 mAbs alone.

The heterogeneity of $CD25^{+}CD4^{+}$ T-cell clones in terms of expression of CD25 and proliferation suggested that even within the 0.6% $CD25^{bright}CD4^{+}$ Tr cell population of PBMCs, not all cells may be suppressor cells, and that a proportion could be activated T helper cells. To test this hypothesis we performed in vitro suppression assays. As shown in Figure 8, indeed only a subset of the $CD25^{+}CD4^{+}$ T cell clones were able to suppress the proliferative response of autologous $CD4^{+}$ T cells in response to αCD3 mAbs cross-linked on T-cell-depleted PBMCs (8A) or immobilized on plastic (8B). Interestingly, only those clones which were anergic and displayed a constitutively high expression of CD25 had a suppressive phenotype. When data from all the $CD25^{+}CD4^{+}$ T-cell clones tested were pooled together, and the clones were separated into suppressive and non-suppressive groups, expression of CD25 was found to be significantly higher in the group with suppressive activity ($p<0.000007$) (Table 2). In contrast, proliferation in response to αCD3 mAbs was a less reliable predictor of suppressive capacity, as several clones within the non-suppressive category were anergic. These data indicate that constitutively high expression of CD25 is a marker for $CD4^{+}$ T regulatory cells at the clonal level.

We also determined the cytokine production profile of the $CD25^{+}CD4^{+}$ T-cell clones. As shown in Table 3, non-suppressive clones tended to possess

a Th0 pattern of cytokine production and produced moderate levels of most cytokines tested. In contrast, all $CD25^+CD4^+$ T-cell clones which had suppressive activity produced significant amounts of TGF-β, small and variable amounts of IL-4, IL-5 and IFN-γ, but failed to produce detectable levels of IL-2 or IL-10. These data indicate that $CD25^+CD4^+$ T regulatory cells are likely distinct from IL-10-producing Tr1 cells, although it cannot be excluded that they represent the same cells at different stages of differentiation. The fact that the only cytokine which was consistently detected in the supernatants of all the suppressive $CD25^+CD4^+$ T-cell clones was TGF-β suggests that they are more likely related to the TGF-β producing Th3 cells which were originally described in models of oral tolerance (27, 28).

TABLES

Table 1. *Cytokine production by $CD25^+CD4^+$ Tr cells*. Purified cells were stimulated as indicated and supernatants were collected after 24 (for IL-2) or 72 hours. The amount of cytokine was determined by ELISA. Data represent the average values (pg/ml) of pooled data from 4 independent experiments. Cytokine production by allogeneic APCs alone has been subtracted.

Stimuli	Cells	IL-2	IL-4	IL-10	IFN-γ	TGF-β
αCD3/28 +IL-2	$CD25^+$	N.D.	153	1148	5723	1322
αCD3/28 +IL-2	$CD25^-$	N.D.	94	840	9773	1225
allogeneic APCs	$CD25^+$	<20	<20	298	527	509
allogeneic APCs	$CD25^-$	99.5	<20	251	5744	637

Table 2. *Suppressive $CD25^+CD4^+$ T-cell clones have a distinct phenotype from non-suppressive clones.* Summary of the phenotype of all the $CD25^+CD4^+$ T-cell clones which were extensively characterized. Percent suppression represents the average reduction of proliferation of autologous $CD4^+$ T cells upon activation with αCD3 mAbs, immobilized on plastic or

T-cell-depleted ACPs, in the presence of the indicated T-cell clones. Numbers represent the average suppression observed in 2-6 independent experiments. MFI represents the average expression of CD25 as determined in 2-6 independent tests. cpm represents the amount of thymidine incorporated following activation with αCD3 mAbs immobilized on plastic. Numbers represent the average of duplicates in a single test, and are representative of results obtained in several subsequent tests.

	suppression (%)	**CD25 MFI**	**αCD3 (cpm)**
non-suppressive			
2	0	36	15947
3	0	83	166
6	0	40	908
37	0	40	36280
84	n.t.	19	20281
85	0	33	1839
86	0	91	23139
87	0	21	4166
88	n.t.	34	36376
89	0	43	17702
90	0	46	21210
92	0	33	34004
93	n.t.	19	20005
94	n.t.	12	6929
95	0	66	410
suppressive			
4	37	90	244
12	40	100	122
13	52	101	511
15	24	211	644
17	36	110	88
18	73	97	123
19	60	88	1289
20	22	98	285
21	76	65	72
22	39	102	180
24	45	174	281
28	45	179	380
29	54	113	98
40	63	85	108
42	45	77	52
47	57	86	61
48	56	68	66
57	31	130	600

Nt: not tested

Table 3. *Cytokine production profile of $CD25^+CD4^+$ T cell clones.* T-cell clones were activated with αCD3 and αCD28 mAbs, and supernatants were collected after 24 (for IL-2) or 48 hours. Amounts of cytokines in the supernatants were determined by capture ELISA and/ or CBA assay as described in the materials and methods. n.t.: not tested.

	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-5 (ng/ml)	IL-10 (pg/ml)	IFN-γ (ng/ml)	TGF-β (pg/ml)
non-suppressive						
2	<20	1184	35.4	79	2.7	181
3	<20	8002	3.5	98	0.8	257
6	<20	57	0.9	<20	0.1	94
37	56	552	3.9	17	7.7	n.t.
84	540	607	4.5	39	7.0	7024
85	<20	521	7.9	94	1.5	214
86	394	537	3.9	62	12.0	n.t.
87	<20	419	3.0	125	4.2	70
88	<20	168	2.5	<20	1.1	n.t.
89	476	604	4.3	101	8.8	n.t.
90	1360	618	4.6	100	12.6	n.t.
92	280	1963	10.2	163	6.7	217
95	<20	46	0.4	169	0.5	329
Suppressive						
4	<20	<20	<0.02	<20	<0.06	31
17	<20	67	0.05	<20	0.07	277
18	<20	<20	<0.02	<20	n.t.	141
19	<20	140	0.3	<20	0.1	311
20	<20	<20	<0.02	<20	<0.06	44
21	<20	<20	<20	<20	<0.06	130
22	<20	<10	<0.02	<20	<0.06	410
29	<20	<20	<0.02	<20	<0.06	182
40	<20	<20	<0.02	<20	<0.06	206
42	<20	83	0.2	<20	0.2	369
57	<20	<10	<0.02	<20	0.1	278

MATERIALS AND METHODS

1. Isolation and characterization of human $CD25^+CD4^+$ Tr cells

Purification of $CD25^+CD4^+$ Tr cells. Human peripheral blood was obtained from healthy donors in accordance with local ethical committee approval. PBMCs were prepared by centrifugation over Ficoll-Hypaque

gradients (Nycomed Amersham, Uppsala, Sweden), and $CD4^+$ T cells were purified by positive or negative selection (by depletion of CD8, CD11b, CD16, CD19, CD36 and CD56 positive cells) with the $CD4^+$ Multisort kit or the Untouched $CD4^+$ T cell Isolation kit, respectively (Miltenyi Biotech, Gladbach, Germany). Following isolation of $CD4^+$ T cells, $CD25^+$ cells were stained with PE-coupled anti-CD25 mAbs and purified following addition of anti-PE coupled magnetic beads (Miltenyi Biotech). Alternatively, $CD4^+$ T cells were purified with magnetic beads directly coupled to anti-CD25 (Miltenyi Biotech) to facilitate FACS analysis. Results obtained with $CD25^+CD4^+$ Tr cells isolated by negative or positive selection, and directly or indirectly coupled CD25 mAbs were identical. Starting with $2x10^8$ PBMCs, typically $2\text{-}3x10^6$ $CD25^+CD4^+$ Tr cells were isolated, with a purity ranging from 90-95%. $CD25^-CD4^+$ T cells were also collected, with a purity ranging from 70-90%. For purification of $CD25^+$ cells following *in vitro* activation of $CD25^-$ cells, $CD25^-CD4^+$ T cells were activated for 48 hours with immobilized anti-CD3 (10μg/ml) and soluble anti-CD28 (1μg/ml) mAbs and $CD25^+$ T cells were purified as described above.

In vitro expansion of T cell lines. $CD25^+CD4^+$ or $CD25^-CD4^+$ T cells were isolated as described. T cells ($2x10^5$ cells/ml) were stimulated with anti-CD3 (1μg/ml) (OKT3, Orthoclone, Jansen Cilag, Italy) in the presence of an allogeneic feeder-cell mixture consisting of $1x10^6$ PBMCs/ml, (irradiated 6000 RADS) and $1x10^5$ JY cells/ml (irradiated 10,000 RADS), an EBV-LCL which expresses high levels of HLA and costimulatory molecules as well as cytokines, as previously described (29, 30). All cultures were performed in X-Vivo 15 medium (BioWhittaker, Bergamo, Italy) supplemented with 10% FCS (Mascia Brunelli, Milan, Italy), 1% pooled human serum, 100 U/ml penicillin/streptomycin (Bristol-Myers Squibb, Sermoneta, Italy) and 2mM glutamine (GibcoBRL, Milan, Italy) (hereafter referred to as complete

medium). Three days after activation, 40U/ml rIL-2 (Chiron Italia, Milan, Italy) was added. Cells were split as necessary and fresh medium with IL-2 was added. T-cell lines were restimulated every 14 days. All experiments on expanded cells were performed at least 10 days after activation.

Proliferation and suppression of T cells. To analyze proliferation in response to polyclonal activation, 96 well round-bottom plates (Costar) were coated overnight at 4°C with anti-CD3 mAbs (10μg/ml) in 0.1M Tris, pH 9.5, and washed three times with PBS. T cells were plated at an initial density of $5x10^5$ cells/ml (100,000 cells/well) in a final volume of 200μl of complete medium in the absence or presence of soluble anti-CD28 mAbs (1μg/ml) (Pharmingen, San Diego, CA), soluble secondary rabbit anti-mouse Abs (10μg/ml) (Sigma, Milan, Italy) and/or IL-2 (100U/ml).

To test antigen-specific T cell proliferation, freshly isolated $CD25^+CD4^+$ Tr or $CD25^-CD4^+$ T cells ($2.5x10^5$ cells/ml) were stimulated with irradiated (6000 Rads) allogeneic PBMCs ($2.5x10^5$ cells/ml) that had been depleted of $CD3^+$ cells by negative selection (Dynal, Oxoid). For suppression, increasing numbers (up to $2.5x10^5$ cells/ml) of freshly isolated autologous $CD25^+CD4^+$ Tr cells were added. Cells were co-cultured in a final volume of 200μl of complete medium in 96 well round-bottom plates. Control IgG (10μg/ml) (Pharmingen), neutralizing anti-IL-10R (10μg/ml) (3F9, Pharmigen) and/or anti-TGF-$\beta_{1,2,3}$ (10μg/ml or 50μg/ml) (R&D), or $F(ab')_2$ anti-CTLA-4 (10μg/ml) (Ancell, Bayport, MN) mAbs were added as indicated. For suppression of memory T cells, cells from expanded $CD25^-CD4^+$ T-cell lines were cultured with allogeneic APCs (from a donor different from that used in the allogeneic feeder-cell mixture), and increasing numbers of expanded autologous $CD25^+CD4^+$ Tr cells were added as described above. For control experiments, $CD25^+CD4^+$ T cells purified from *in vitro*-activated $CD25^-CD4^+$ T cells were added in increasing numbers to freshly isolated autologous

$CD25^-CD4^+$ T cells and allogeneic APCs.

After the indicated time, wells were pulsed for 16 hours with 1μCi/well ^{3}H-thymidine (Amersham, Uppsala, Sweden). Cells were harvested, and counted in a scintillation counter.

ELISAs. For detection of IL-10, IL-4, IL-2, IFN-γ, and TGF-β, capture ELISAs were performed on supernatants of cells ($1x10^6$ T cells/ml) that had been stimulated with immobilized anti-CD3 mAbs (10μg/ml) with or without anti-CD28 (1μg/ml) and IL-2 (100U/ml), or irradiated CD3-depleted allogeneic PBMCs ($1x10^6$ cells/ml) for 24 (for IL-2) or 72 hours. ELISAs were performed according to the manufacturer's instructions. All capture and detection mAbs were purchased from Pharmingen. The limits of detection were as follows: IL-2: 19pg/ml; IL-4: 9.4pg/ml; IL-10: 15.6pg/ml; IFN-γ: 62.5pg/ml; TGF-β: 62.5 pg/ml.

FACS analysis. Anti-CD4, -CD25, -HLA-DR, -CD45RO, -CD62L, -CD69 and -CD40L mAbs were purchased from Beckton Dickinson (Mountain View, CA) and were directly coupled to FITC or PE. Expression of IL-2Rβ (CD122) and IL-2Rγ (CD132) was determined by staining with the relevant biotinylated mAbs (PharMingen) and streptavidin-coupled TriColor (Caltag). Cells that were resting, or that had been activated with immobilized anti-CD3 (10μg/ml), or PMA (10ng/ml, Sigma) and calcium ionophore (A23187, 500ng/ml, Sigma), were incubated with the indicated mAbs for 20 mins at 4^0C in PBS, 2% FCS, washed once and analyzed with a FACScan® flowcytometer using Cellquest software (Beckton Dickinson). Expression of CTLA-4 was determined by intracytoplasmic staining with biotinylated anti-CTLA-4 (PharMingen) followed by streptavidin-coupled PE (Caltag). Resting or activated cells were fixed with 2% formaldehyde, and membranes were permeabilized by incubation in saponin buffer (PBS, 2% BSA and 0.5% saponin (Sigma)) for 10 mins. Staining and washing were performed in

saponin buffer, and cells were washed once in PBS, 2% BSA prior to analysis.

2. Isolation and characterization of human $CD25^+CD4^+$ Tr cell clones

Purification and cloning of $CD25^+CD4^+$ Tr cells. $CD4^+$ T cells from PBMCs were obtained as described above. $CD4^+$ T cells were stained with FITC-coupled anti-CD4 and PE-coupled anti-CD25 mAbs (Beckton-Dickson) and $CD25^+$ and $CD25^-$ cells were purified by FACS-sorting on a FACStar (Beckton-Dickson). $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells were subsequently cloned at 1 cell/well in 96-well round bottom plates by limiting dilution in the presence of an allogeneic feeder-cell mixture consisting of $5x10^5$ PBMCs/ml, $5x10^4$ JY cells/ml and 0.05µg/ml PHA. After 3 days, IL-2 (40 U/ml) was added. Tcell clones were cultured in X-vivo 15 with 5% Human Serum. At day 14, growing wells were picked and re-stimulated with an allogeneic feeder-cell mixture as described above. Clones were split as necessary, and restimulated as above every 14 days. The medium was replenished every 3-5 days. Clones were used for experiments between days 10 and 14 of the previous re-stimulation (i.e. in the resting phase).

Proliferation and suppression of T cells. To analyze the proliferative capacity of T-cell clones in response to polyclonal activation, 96 well round-bottom plates (Costar) were coated overnight at 4°C with anti-CD3 mAbs (10µg/ml) in 0.1M Tris, pH 9.5, and washed three times with PBS. T-cell clones were plated at an initial density of $2x10^5$ cells/ml (40,000 cells/well) in a final volume of 200µl of complete medium in the absence or presence of IL-2 (100U/ml). To test for the capacity of T-cell clones to suppress the proliferation of autologous $CD4^+$ T cells, fresh $CD4^+$ T cells were purified by positive selection (Miltenyi Biotech) and stimulated with anti-CD3 mAbs which had been immobilized on plastic (as described above) or bound to allogeneic CD3-depleted PBMCs (irradiated 6000 RADS). $CD4^+$ T cells

(40,000 cells/well) were cultured alone, or in the presence of a 1:1 ratio of T-cell clones in a final volume of 200µl of complete medium in 96 well round-bottom plates.

After the indicated time, wells were pulsed for 16 hours with 1µCi/well 3H-thymidine (Amersham, Uppsala, Sweden). Cells were harvested, and counted in a scintillation counter.

ELISAs. T cell clones ($1x10^6$ cells/ml) were stimulated with immobilized anti-CD3 mAbs (10µg/ml) and anti-CD28 (1µg/ml), and supernatants were collected after 24 hours for IL-2 and after 48 hours for all other cytokines. Levels of TGF-β in acidified supernatants were determined by capture ELISA as described above. Levels of IL-2, IL-4, IL-5, IL-10 and IFN-γ were either determined either by capture ELISA (BD Biosciences) as described above or by the cytometric bead array kit (CBA) (BD Biosciences), according to the manufacture's instructions. A direct comparison of capture ELISA and CBA demonstrated that the two methods were highly comparable in terms of the amount of cytokine detected in the supernatant.

Statistical Analysis. All analysis for statistically significant differences were performed with the student's paired *t* test. *p* values less than 0.05 were considered significant. Results are expressed as means ± SEM.

Abbreviations

CD cluster of differentiation

IL interleukin

TGF transforming growth factor

APC antigen presenting cell

mAb monoclonal antibody

FACS fluorescence activated cell sorting

PHA phytohemoagglutinin

PBMC peripheral blood mononuclear cells

MFI mean fluorescence intensity

EBV Ebstein-Barr virus

REFERENCES

1. Roncarolo, M.G., and M.K. Levings. 2000. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr. Opin. Immunol.* 12:676-83.

2. Sakaguchi, S. 2000. Regulatory T cells: Key Controllers of Immunologic Self-Tolerance. *Cell* 101:455-8.

3. Shevach, E.M. 2000. Regulatory T cells in autoimmmunity. *Annu Rev Immunol* 18:423-49.

4. Cottrez, F., S.D. Hurst, R.L. Coffman, and H. Groux. 2000. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J. Immunol.* 165:4848-53.

5. Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med* 190, no. 7:995-1004.

6. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389, no. 6652:737-42.

7. Powrie, F., J. Carlino, M.W. Leach, S. Mauze, and R.L. Coffman. 1996. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) $CD4^+$ T cells. *J Exp Med* 183, no. 6:2669-74.

8. Chen, Y., V.K. Kuchroo, J. Inobe, D.A. Hafler, and H.L. Weiner. 1994. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265, no. 5176:1237-40.

9. Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12, no. 4:431-40.

10. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T Lymphocyte-

associated Antigen 4 Plays an Essential Role in the Function of CD25(+)CD4(+) Regulatory Cells that Control Intestinal Inflammation. *J Exp Med* 192, no. 2:295-302.

11. Suri-Payer, E., A.Z. Amar, A.M. Thornton, and E.M. Shevach. 1998. $CD4^+CD25^+$ T cells inhibit both the induction and effector function of autoreactive T cells and represent a unique lineage of immunoregulatory cells. *J Immunol* 160, no. 3:1212-8.

12. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184:387-96.

13. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 162, no. 9:5317-26.

14. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-64.

15. Thornton, A.M., and E.M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188, no. 2:287-96.

16. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int Immunol* 10, no. 12:1969-80.

17. Thornton, A.M., and E.M. Shevach. 2000. Suppressor effector function

of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J Immunol* 164, no. 1:183-90.

18. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T.W. Mak, and S. Sakaguchi. 2000. Immunologic Self-Tolerance Maintained by CD25(+)CD4(+) Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte-associated Antigen 4. *J Exp Med* 192, no. 2:303-310.

19. Cederbom, L., H. Hall, and F. Ivars. 2000. $CD4^+CD25^+$ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* 30:1538-43.

20. Kitani, A., K. Chua, K. Nakamura, and W. Strober. 2000. Activated Self-MHC-Reactive T Cells Have the Cytokine Phenotype of Th3/T Regulatory Cell 1 T Cells. *J Immunol* 165, no. 2:691-702.

21. Kuniyasu, K., T. Takahashi, M. Itoh, J. Shimizu, G. Toda, and S. Sakaguchi. 2000. Naturally anergic and suppressive CD25(+)CD4(+) T cells as a functionally and phenotypically distinct immunoregulatory T cell population. *Int Immunol* 12:1145-55.

22. Roncarolo, M.G., R. Bacchetta, C. Bordignon, S. Narula, and M.K. Levings. 2001. Type 1 T regulatory cells. *Immunol Rev* 182:68-79.

23. Shevach, E.M., R.S. McHugh, C.A. Piccirillo, and A.M. Thornton. 2001. Control of T-cell activation by $CD4^+$ $CD25^+$ suppressor T cells. *Immunol Rev* 182:58-67.

24. Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev* 182:18-32.

25. Baecher-Allan, C., J.A. Brown, G.J. Freeman, and D.A. Hafler. 2001.

$CD4^+CD25$high regulatory cells in human peripheral blood. *J Immunol* 167, no. 3:1245-53.

26. Levings, M.K., R. Sangregorio, and M.G. Roncarolo. 2001. Human $CD25^+CD4^+$ T regulatory cells suppress naive and memory T-cell proliferation and can be expanded *in vitro* without loss of function. *J. Exp. Med.* 193:1295-1302.

27. Chen, Y., J. Inobe, and H.L. Weiner. 1995. Induction of oral tolerance to myelin basic protein in CD8-depleted mice: both $CD4^+$ and $CD8^+$ cells mediate active suppression. *J Immunol* 155, no. 2:910-6.

28. Weiner, H.L. 2001. Induction and mechanism of action of transforming growth factor-beta- secreting Th3 regulatory cells. *Immunol Rev* 182:207-14.

29. Bacchetta, R., M. Bigler, J.L. Touraine, R. Parkman, P.A. Tovo, J. Abrams, R. de Waal Malefyt, J.E. de Vries, and M.G. Roncarolo. 1994. High levels of interleukin 10 production in vivo are associated with tolerance in SCID patients transplanted with HLA mismatched hematopoietic stem cells. *J Exp Med* 179, no. 2:493-502.

30. van de Griend, R.J., and R.L. Bolhuis. 1984. Rapid expansion of allospecific cytotoxic T cell clones using nonspecific feeder cell lines without further addition of exogenous IL2. *Transplantation* 38:401-6.

31. Taylor PA, Lees CJ, Blazar BR. The infusion of ex-vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality. Blood 2002 May 15; 99(10): 3493-9

32. Gould DS, Auchincloss H Jr. Direct and indirect recognition: the role of MHC antigens in graft rejection. Immunol. Today, 1999 Feb; 20(2):77-82.

CLAIMS

1. The use of ex-vivo isolated human $CD25^+CD4^+$ Tr cells for the preparation of immunomodulating or immunosuppressive agents.

2. The use of ex-vivo isolated human $CD25^+CD4^+$ Tr cell clones constitutively expressing CD25 for the preparation of immunomodulating or immunosuppressive agents.

3. The use according to claim 1 or 2, for the prevention or therapy of graft-vs-host disease, organ rejection, autoimmune diseases and for the prevention of adverse immune responses to transgenes and vector-derived proteins after gene therapy.

4. The use according to claim 1, wherein human $CD25^+CD4^+$ Tr cells are freshly isolated from PBMC or expanded in-vitro.

5. The use according to claim 2, wherein the $CD25^+CD4^+$ Tr cell clones constitutively expressing CD25 are isolated ex-vivo by the following steps:

a) purifying $CD4^+$T cells from PBMCs;

b) separating $CD25^+$ from $CD25^-$ T cells;

c) cloning $CD25^+CD4^+$ T cells by limiting dilution;

d) stimulation with phytohemagglutinin or anti-CD3 mAb, in the presence of IL-2;

e) selecting the suppressive clones that display a constitutively high expression of CD25.

6. An immunosuppressive agent containing the isolated human $CD25^+CD4^+$ Tr cells or $CD25^+CD4^+$ Tr cell clones constitutively expressing CD25.

7. An immunosuppressive agent according to claim 6, further containing cytokines or additional immunosuppressants.

8. An immunosuppressive agent according to claims 6-7, which is in form

of stabilized cell preparation.

9. A method of isolating immunosuppressive $CD25^+CD4^+$ Tr cell clones which comprises the steps of:

a) purifying $CD4^+$T cells from PBMCs;

b) separating $CD25^+$ from $CD25^-$ T cells;

c) cloning $CD25^+CD4^+$ T cells by limiting dilution;

d) stimulation with phytohemagglutinin or anti-CD3 mAb, in the presence of IL-2;

e) selecting the suppressive clones that display a constitutively high expression of CD25.

10. A method according to claim 9, wherein the stimulation according to step (d) is carried out in the presence of an allogenic or autologous feeder-cell mixture consisting of irradiated PBMCs.

11. A method according to claim 10, which is carried out with irradiated autologous or allogeneic EBV-transformed cell lines.

12. A method according to claim 9, wherein in step (d) the suppressive clones are selected on the basis of the following characteristics:

- 100% constant-positivity for CD25 expression in the resting phase at least 10 days after stimulation with phytohemagglutinin or anti-CD3 mAb in the presence of IL-2;
- expression of CD25 at a significantly higher level in comparison to T cell clones isolated in parallel from $CD25^-CD4^+$ T cells or non suppressive clones isolated from $CD25^+CD4^+$ T cells.

13. Isolated $CD25^+CD4^+$ Tr cell clones obtainable by the process of claims 9-11.

14. Isolated $CD25^+CD4^+$ Tr clones according to claim 12, which do not produce IL-2.

15. The use of $CD25^+CD4^+$ Tr cell clones according to claims 12-13 for the

preparation of in vitro systems for the identification of molecules that modulate the immune response.

16. The use according to claim 15, in large scale gene expression arrays, differential proteomics screenings and for the generation of monoclonal antibodies.

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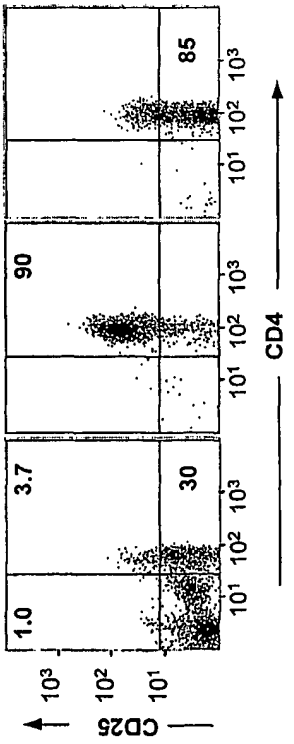
Figure 1 A



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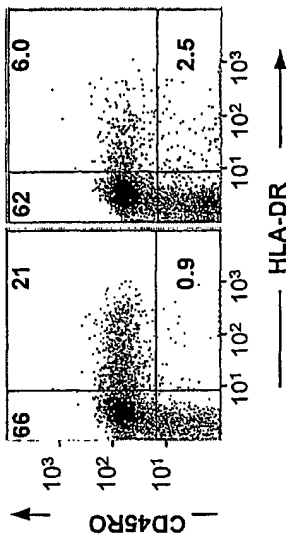

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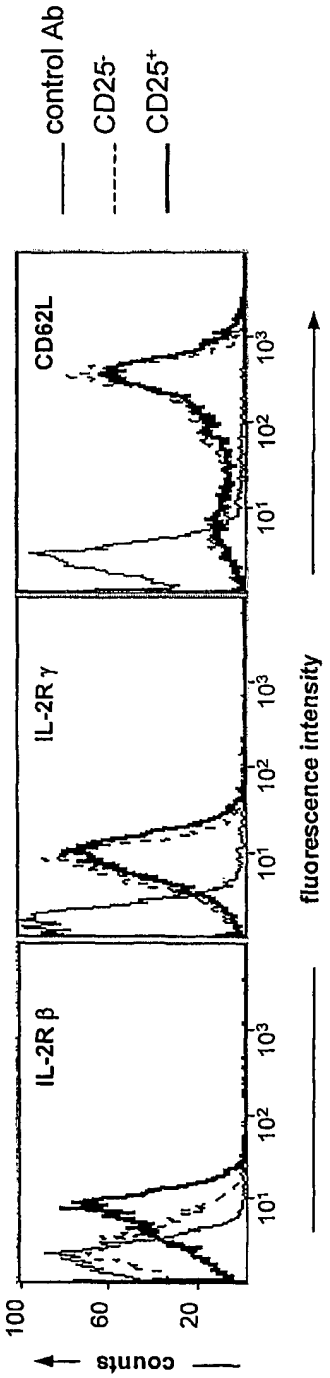

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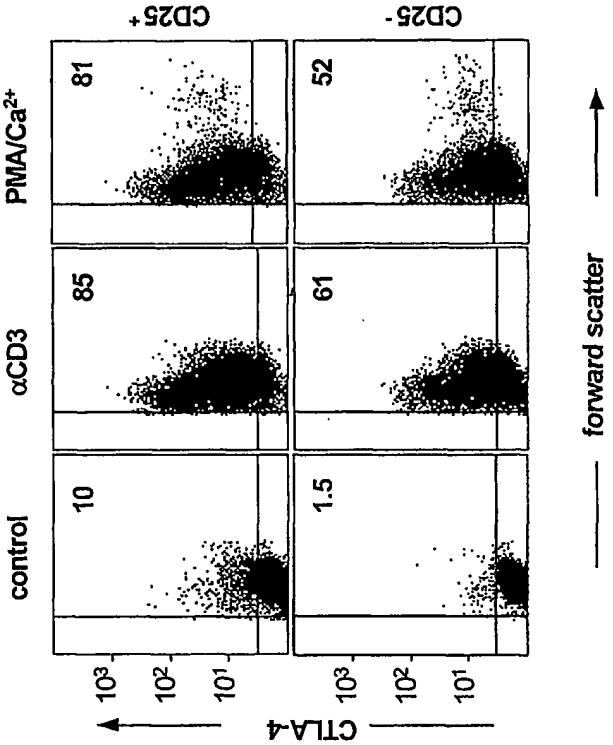

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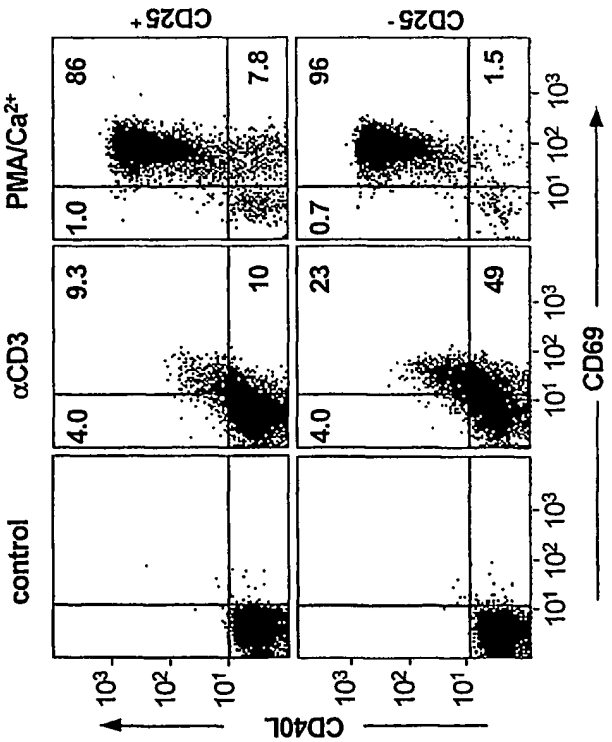

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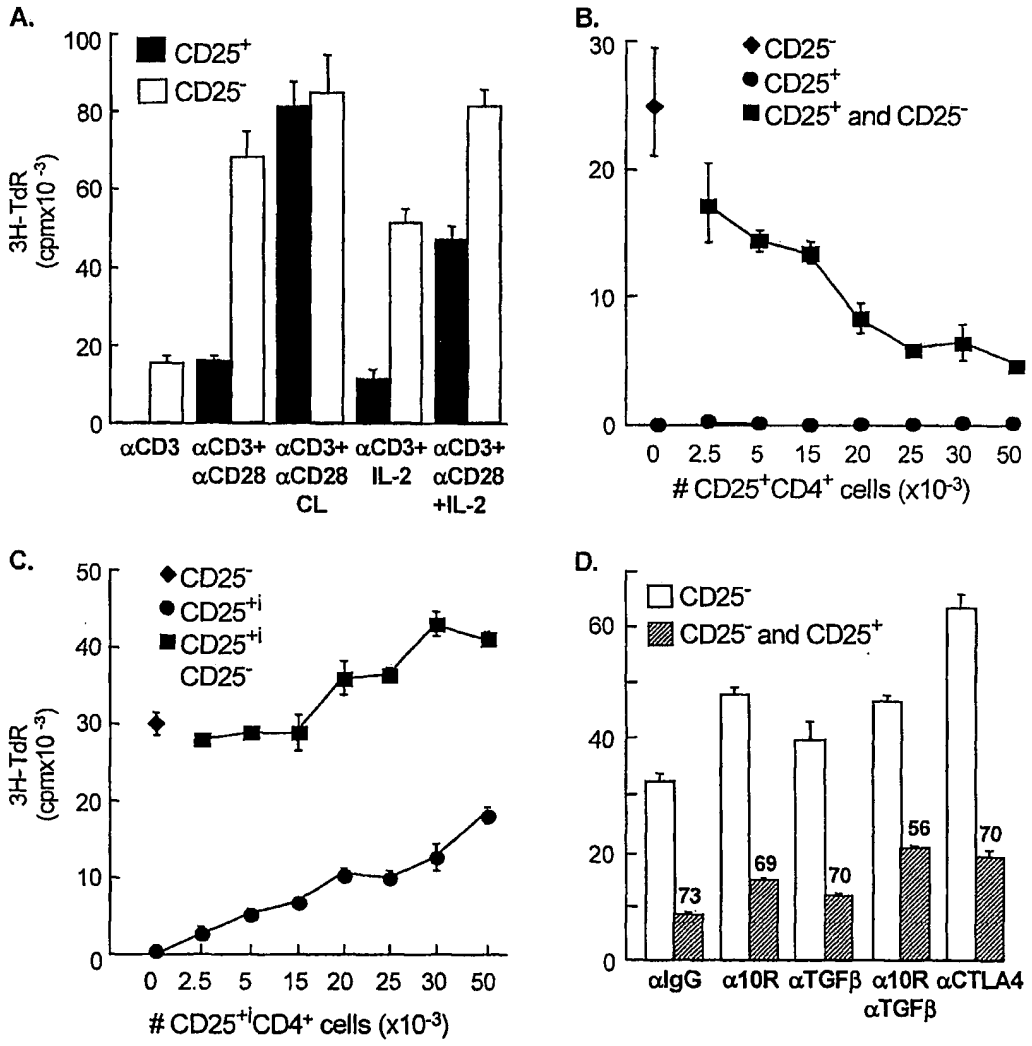

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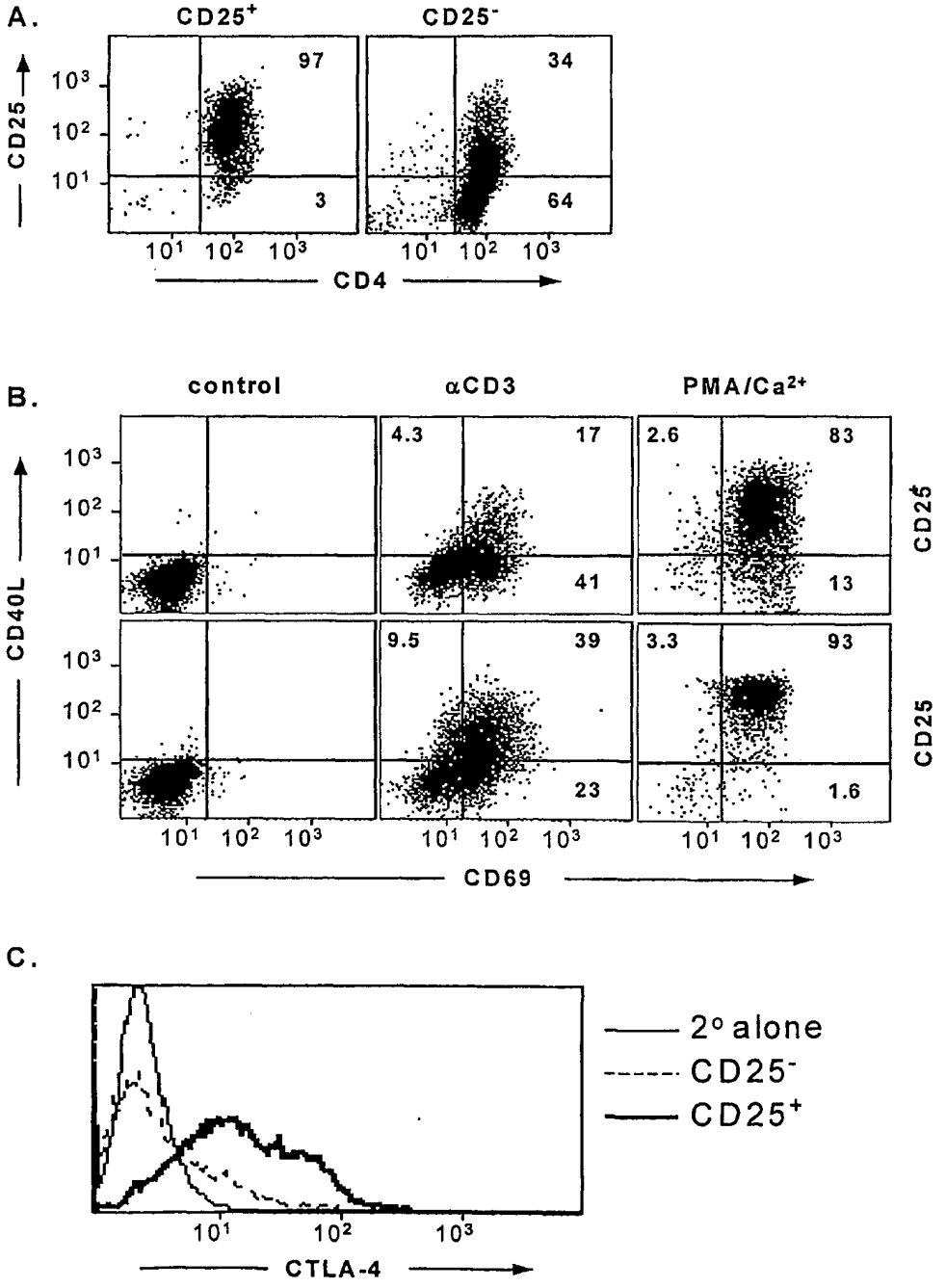

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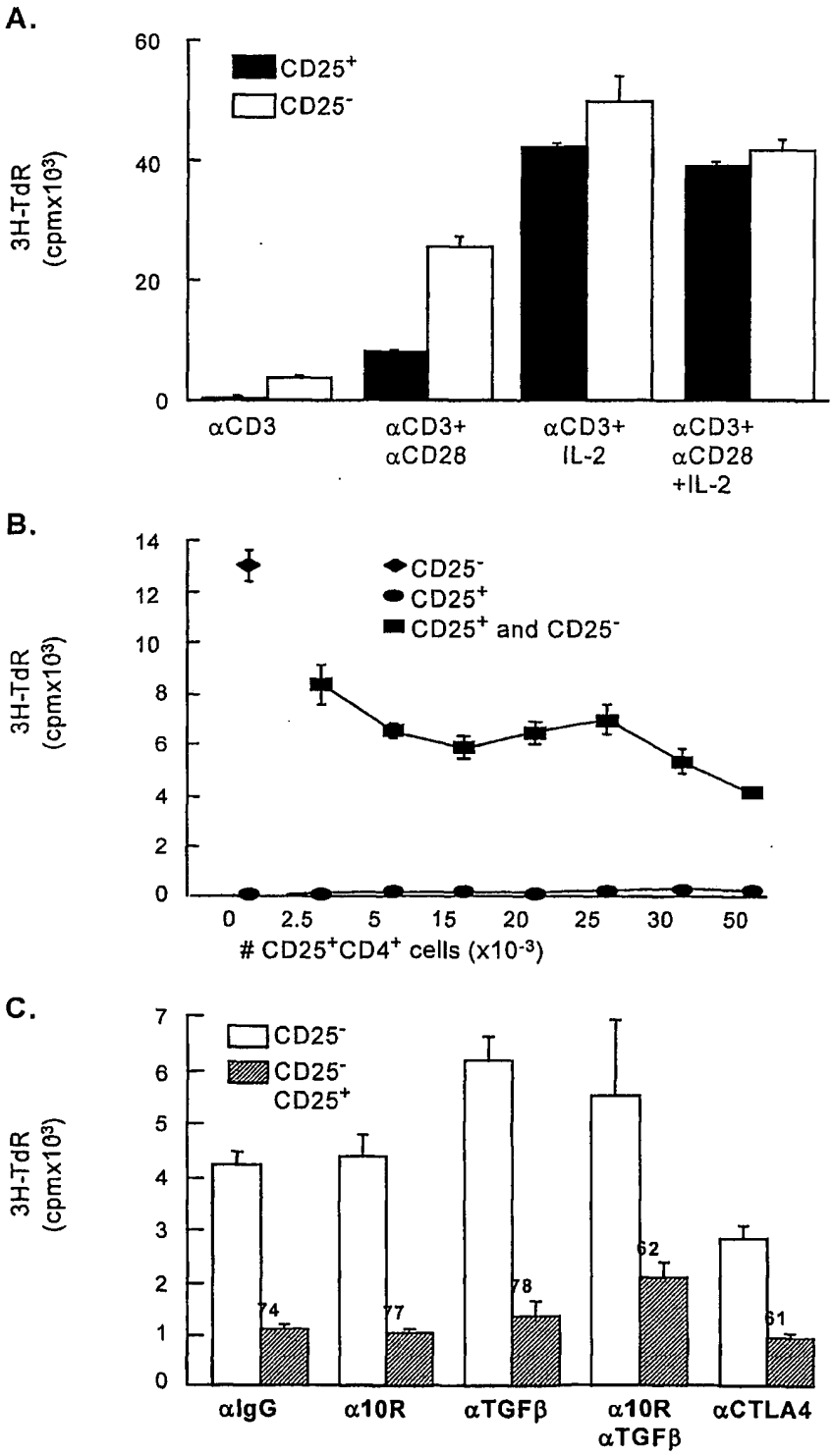
Figure 4



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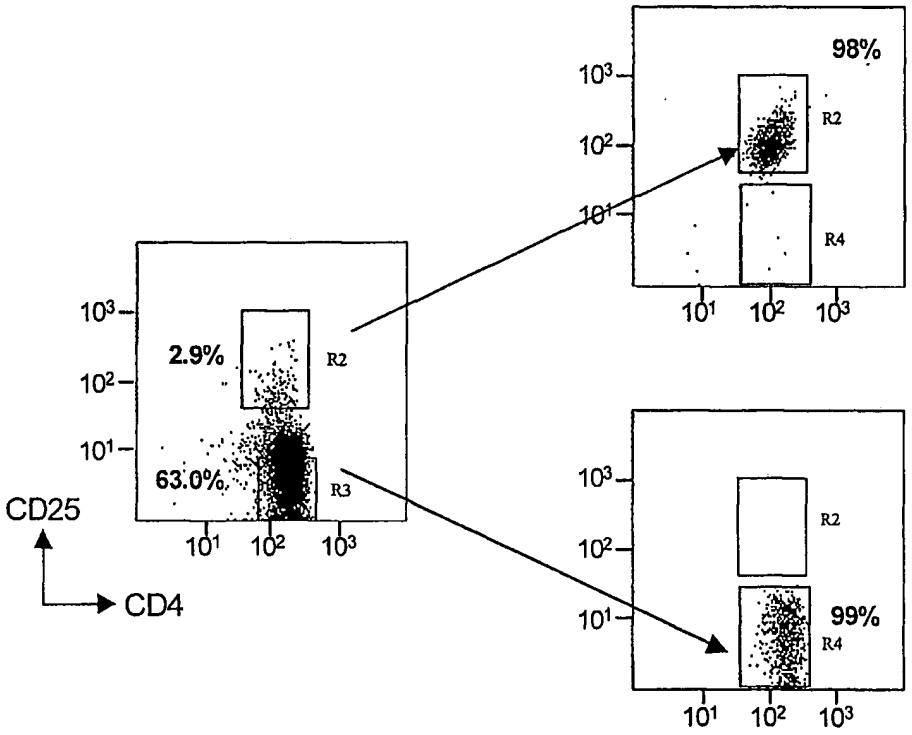

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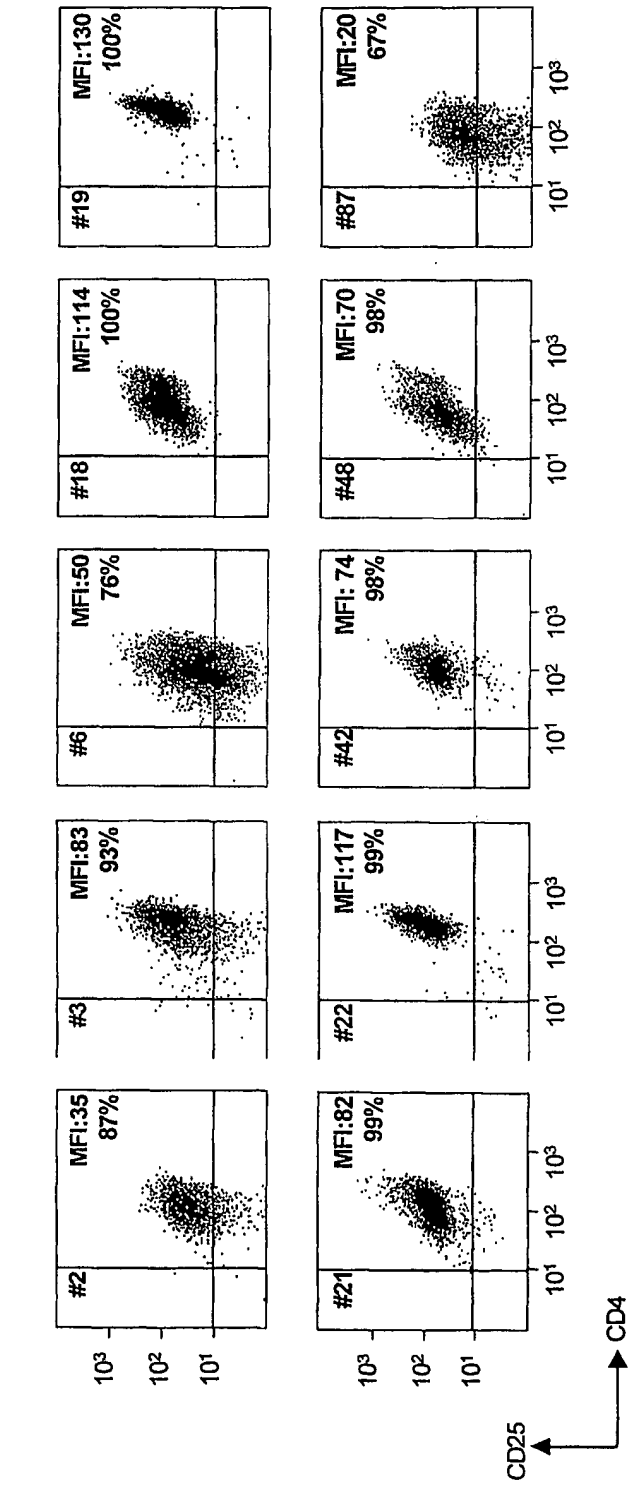
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Figure 7

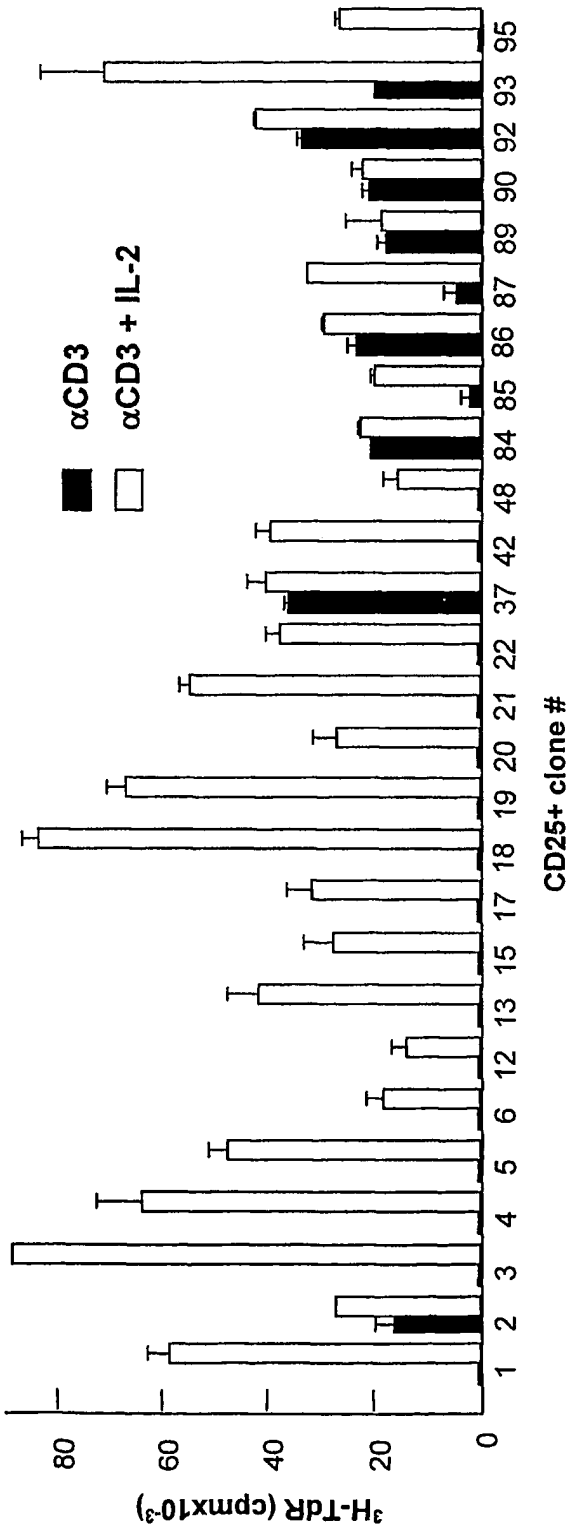

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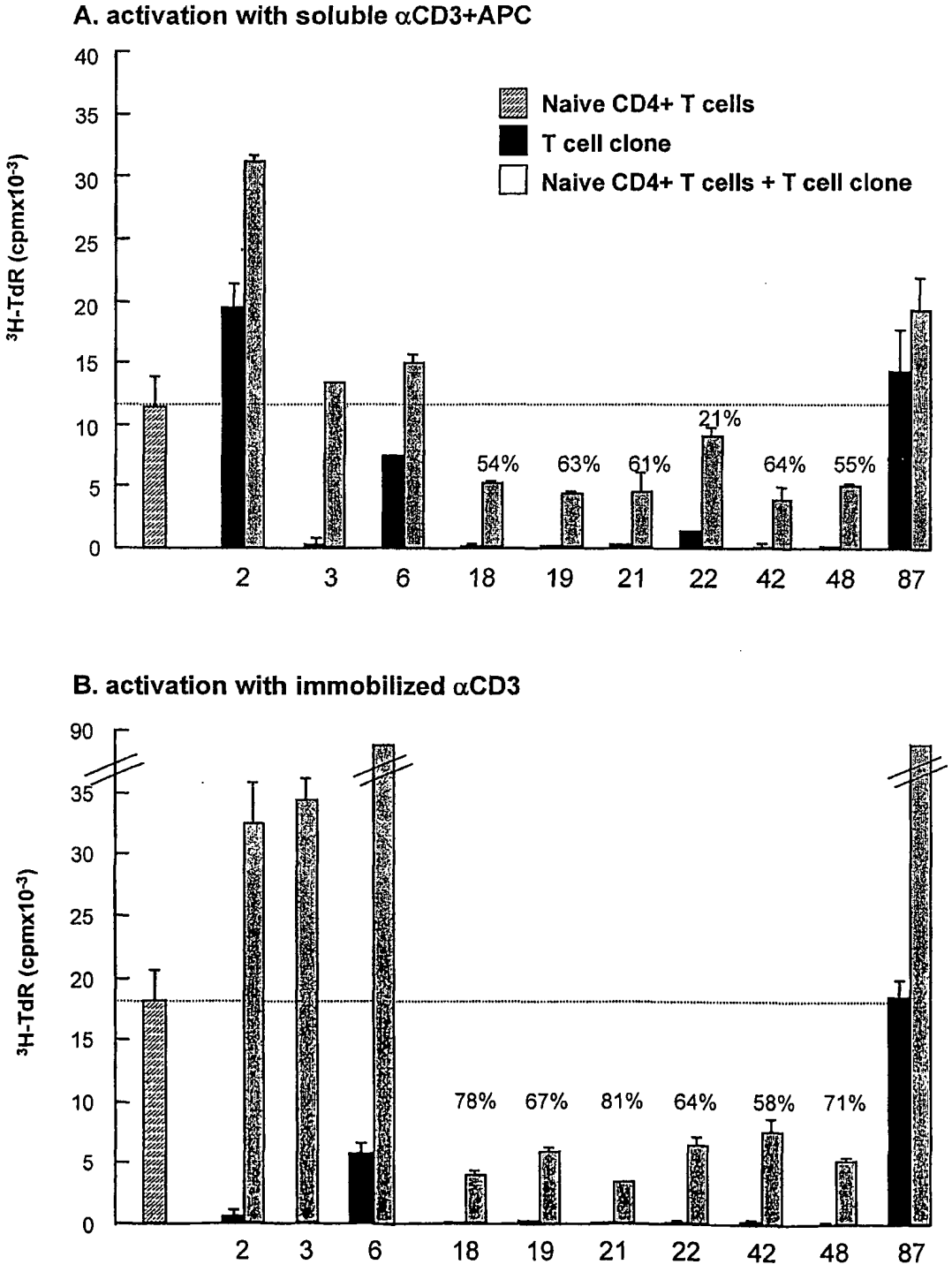
Figure 8



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/05919

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/08 A61K35/14

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEPHENS LEIGH A ET AL: "Human CD4+CD25+ thymocytes and peripheral T cells have immune suppressive activity in vitro." EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 31, no. 4, April 2001 (2001-04), pages 1247-1254, XP002210570 ISSN: 0014-2980	1,4,6-8
Y	the whole document	2,3,5, 9-16

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
22 August 2002	11/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Friedrich, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RONCAROLO M-G ET AL: "The role of different subsets of T regulatory cells in controlling autoimmunity" CURRENT OPINION IN IMMUNOLOGY, CURRENT BIOLOGY LTD, XX, vol. 12, no. 6, 1 December 2000 (2000-12-01), pages 676-683, XP004257742 ISSN: 0952-7915 the whole document	2,3,5, 9-16
P,X	LEVINGS MEGAN K ET AL: "Human CD25+CD4+ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 193, no. 11, 4 June 2001 (2001-06-04), pages 1295-1301, XP002210571 ISSN: 0022-1007 the whole document	1-16